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 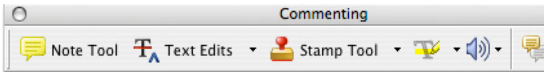

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 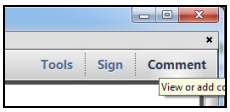

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 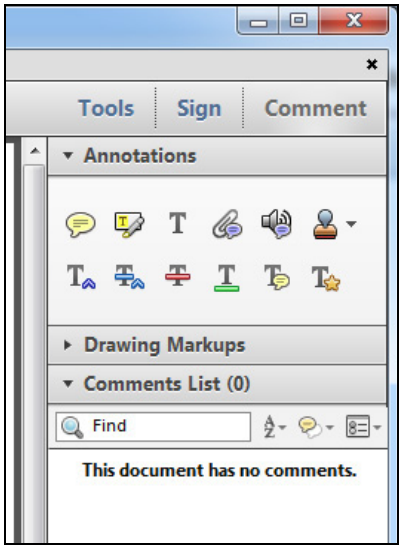
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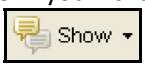
Review	To review your changes, click on the 'Show' button  on the Commenting tool bar. Choose 'Show Comments List'. Navigate by clicking on a correction in the list. Alternatively, double click on any mark-up to open the commenting box.	Your changes will appear automatically in a list below the Comment tool bar. Navigate by clicking on a correction in the list. Alternatively, double click on any mark-up to open the commenting box.
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Duodenal and Rectal Mucosa Inflammation in Patients With Non-celiac Wheat Sensitivity

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BACKGROUND & AIMS: **Studies of non-celiac gluten or wheat sensitivity (NCGWS) have increased but there are no biomarkers of this disorder. We aimed to evaluate histologic features of colon and rectal tissues from patients with NCGWS.**

METHODS: **We performed a prospective study of 78 patients (66 female; mean age, 36.4 years) diagnosed with NCGWS by double-blind wheat challenge at 2 tertiary care centers in Italy, from January 2015 through September 2016. Data were also collected from 55 patients wither either celiac disease or self-reported NCGWS but negative results from the wheat-challenge test (non-NCGWS controls). Duodenal and rectal biopsies were collected and analyzed by immunohistochemistry to quantify intra-epithelial $CD3^+$ T cells, lamina propria $CD45^+$ cells, $CD4^+$ and $CD8^+$ T cells, mast cells, and eosinophils and to determine the presence and size of lymphoid nodules in patients with NCGWS vs patients with celiac disease or non-NCGWS controls.**

RESULTS: **Duodenal tissues from patients with NCGWS had significantly higher numbers of intra-epithelial $CD3^+$ T cells, lamina propria $CD45^+$ cells, and eosinophils than duodenal tissues from non-NCGWS controls. Duodenal tissues from patients with NCGWS and dyspepsia had a higher number of lamina propria eosinophils than patients with NCGWS without upper digestive tract symptoms. Rectal mucosa from patients with NCGWS had a larger number of enlarged lymphoid follicles, intra-epithelial $CD3^+$ T cells, lamina propria $CD45^+$ cells, and eosinophils than rectal mucosa from non-NCGWS controls. Duodenal and rectal mucosal tissues from patients with celiac disease had more immunocytes ($CD45^+$ cells, $CD3^+$ cells, and eosinophils) than tissues from patients with NCGWS or non-NCGWS controls.**

CONCLUSIONS: **We identified markers of inflammation, including increased numbers of eosinophils, in duodenal and rectal mucosa from patients with NCGWS. NCGWS might therefore involve inflammation of the entire intestinal tract. Eosinophils could serve as a biomarker for NCGWS and be involved in its pathogenesis. Clinicaltrials.gov: NCT01762579.**

Keywords: Bread; Food Allergy; Irritable Bowel Syndrome; Nonceliac Wheat Sensitivity; Histology.

Nonceliac gluten/wheat sensitivity (NCGWS) is characterized by intestinal (ie, bloating, dyspepsia) and extraintestinal symptoms (ie, fatigue, headache) following ingestion of gluten-containing food in subjects without celiac disease (CD) or wheat allergy.[1] Because NCGWS is triggered by gluten or wheat ingestion, CD must be excluded before a diagnosis of NCGWS can be confirmed.[1] Consequently, duodenal histology, lack of villus atrophy, and evaluation of intraepithelial infiltration of the duodenal mucosa have been considered fundamental steps in the diagnostic work-up of NCGWS.

However, many patients with NCGWS have symptoms overlapping with irritable bowel syndrome (IBS),[2] and patients with IBS have also been shown to benefit from a gluten-free diet.[3–6] The pathogenesis of IBS is complex

Abbreviations used in this paper: CD, celiac disease; DBPC, double-blind placebo-controlled; IBS, irritable bowel syndrome; IEL, intraepithelial lymphocytes; NCGWS, nonceliac gluten/wheat sensitivity.

1542-3565/$36.00
https://doi.org/10.1016/j.cgh.2018.08.043

and incompletely understood. Peripheral and central mechanisms can cause the alterations of gastrointestinal motor and sensory functions seen in IBS.[7] Alterations of the mucosal immune system are believed to play a role in IBS and some patients may indeed have inflammation of the colonic mucosa.[8] Consequently, it is logical to study the colon of patients with NCGWS for possible inflammation in this site.

On this basis, we designed the present study (1) to search for the presence of mucosal inflammation in the rectum of patients with NCGWS; (2) to compare the presence, entity, and cell composition of inflammation in the duodenal and rectal mucosa of patients with NCGWS; and (3) to compare the rectal and duodenal mucosal inflammation of patients with NCGWS and control subjects to identify possible distinctive markers of NCGWS.

What You Need to Know

Background

There are no markers of non-celiac wheat sensitivity. Its clinical presentation can be similar to that of irritable bowel syndrome, but no studies have evaluated histologic features of rectal biopsies from these patients.

Findings

Duodenal and rectal mucosa biopsies from patients with non-celiac wheat sensitivity had a higher number of immune cells and eosinophils than tissues from controls. Eosinophil infiltration was more prominent in rectal vs duodenal tissues of patients with non-celiac wheat sensitivity.

Implications for patient care

Evaluation of patients for non-celiac wheat sensitivity should include histologic analysis of rectal biopsies. Eosinophil infiltration of the rectal mucosa, in absence of endoscopic findings, could be a marker of non-celiac wheat sensitivity.

Methods

Patients

We prospectively recruited consecutive adult patients with self-reported wheat sensitivity. from 2 Italian tertiary centers (Department of Internal Medicine, University Hospital of Palermo, and Department of Internal Medicine, Hospital of Sciacca) between January 2015 and September 2016. Patients were referred for gastroenterologic symptoms with a self-reported onset that could be related to wheat ingestion. During this period, 522 patients with suspected NCGWS were studied.

Exclusion criteria were: (1) age <18 years, (2) self-exclusion of wheat from the diet and refusal to reintroduce it before entering the study, (3) steroids or nonsteroidal anti-inflammatory drugs in the 2 weeks before endoscopic investigation, (4) presence of other "organic" gastrointestinal diseases, (5) pregnancy, (6) infectious diseases, and (7) immunologic deficiency.

Nonceliac Gluten/wheat Sensitivity Diagnosis

All the patients met the following criteria: negative test for serum antitransglutaminase and antiendomysium IgA and IgG antibodies, absence of intestinal villous atrophy, and absence of wheat allergy based on a negative IgE-mediated immune-allergy test (skin prick tests and/or serum-specific IgE detection). Other criteria were resolution of symptoms on a standard oligoantigenic diet (ie, excluding wheat, cow's milk, egg, tomato, chocolate, or other foods causing self-reported symptoms), and symptom reappearance with the double-blind placebo-controlled (DBPC) wheat challenge (see later).

Exclusion of Other Diagnoses in Nonceliac Gluten/wheat Sensitivity Patients

CD, IgE-mediated wheat allergy, and inflammatory bowel diseases were carefully excluded in accordance with current recommendations, as previously described[9] (for details see Supplementary Appendix 1).

Elimination Diet and Double-blind Placebo-controlled Challenge

These were performed as previously described.[9] In brief, the patient commenced an oligoantigenic diet, excluding wheat, cow's milk, and other foods, and subsequently underwent DBPC wheat challenge, using 80 g of wheat flour (6.5 g of gluten) or rice flour (as placebo) for 2 weeks, with a cross-over design; the 2 flour types were given in sachets and consumed after cooking and there was no distinguishable difference in their appearance (for details see Supplementary Appendix 1).

Control Subjects

We included 2 different groups of patients recruited in the same centers as control subjects. The first group was composed of 39 patients (30 women, 9 men; mean age, 36.2 years), who self-reported gastrointestinal and/or extraintestinal symptoms after eating wheat (exactly like the NCGWS group) but who did not respond to the DBPC challenge (25 of them also reacted to placebo and 14 did not react to wheat). They were recruited during the study period and had similar sex and age to the patients with NCGWS. The second control group included 16 patients (14 women, 2 men; mean age, 36.1 years) with CD, with sex and age similar to the patients with NCGWS and chosen at random from those diagnosed with CD during the study period.

Intestinal Biopsies

Both duodenal and rectal biopsies, oriented on acetate cellulose filters, were performed in patients with NCGWS and in non-NCGWS control subjects after they had consumed a wheat-containing diet (a minimum of 100 g) for at least 4 weeks. At this time, all patients were symptomatic and reported the symptoms included in Table 1. During the 4-week wheat reintroduction period, the patients avoided other foods that they self-reported as causing symptoms. CD control subjects underwent only duodenal biopsies while they were on the gluten-containing diet.

In all cases at least 4 mucosal specimens were taken from the duodenal bulb and the second part of the duodenum, during esophagogastroduodenoscopy. Multiple rectal biopsies were taken at 5–15 cm from the anal verge, during proctoscopy.

Duodenal Histology Study

The following parameters were evaluated: villus/crypt ratio, presence of infiltration in the lamina propria, presence of crypt distortion, number of intraepithelial $CD3^+$ lymphocytes (IEL) per 100 enterocytes. Furthermore, $CD45^+$ immunocytes, $CD3^+$ lymphocytes, $CD4^+$ and $CD8^+$ lymphocytes, mast cells, and eosinophils in the lamina propria were counted.

Rectal Histology Study

The following parameters were evaluated: presence of crypt distortion, presence of lymphoid nodules, number and size of lymphoid nodules, and number of IEL. Furthermore, in the lamina propria we counted the number of $CD45^+$ immunocytes; $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes; tryptase-positive cells (mast cells); and eosinophils.

Biopsy specimens were assessed in Palermo by 2 of the authors (G.G. and/or A.M.F.); the eosinophil count was further assessed by an experienced gastrointestinal pathologist (V.V.) in Brescia. All reviewers were blinded to the diet allocation and final diagnosis of each patient. For the method details, see Supplementary Appendix 2.

Statistical Analysis

Data were expressed as mean ± standard deviation when the distribution was Gaussian, and differences were calculated using Student t test. Otherwise, data were expressed as median and range and analyzed with the Mann-Whitney U test. Fisher exact test or the chi-square test was used where appropriate. The Mantel-Haenszel test was used to compare the severity of duodenal and rectal histology damage in the different patient groups studied.

To compare the severity of lamina propria eosinophil infiltration in the duodenum and rectum of the patients with NCGWS, values were expressed as fold increase over the upper limit for our laboratory, and the Mann-Whitney U test was used. In the duodenum, the upper limit of the reference interval in our laboratory was 40 lamina propria eosinophils per 10 high-power fields. In the rectal biopsy specimens, the upper limit of the reference interval was <9 lamina propria eosinophils per 5 high-power fields.

To assess agreement between the pathologists in evaluating the lamina propria eosinophil infiltration, Cohen-Fleiss k coefficient values were calculated. This test was applied as the "presence/absence of eosinophil infiltration," referring to the refence interval in our laboratory (for details, see Supplementary Appendix 2).

All data were analyzed using SPSS version 22.0 (SPSS Inc, Chicago, IL) and MedCalc (MedCalc Software, Mariakerke, Belgium). The study protocol conformed to the ethical guidelines of the Declaration of Helsinki. It was approved by the Human Research Committee of the University of Palermo and registered at clinicaltrials.gov (registration number: NCT01762579, "Bio-markers of

Table 1. Demographic and Clinical Characteristics of 78 Patients With NCGWS, in 39 Self-reported NCGWS Subjects Negative at the Wheat Challenge (Non-NCGWS Control Subjects) and in 16 Patients With CD

	NCGWS patients (n = 78)	Non-NCGWS control subjects (n = 39)	CD (n = 16)
Age, mean y + SD	36.4 ± 11.6	36.2 ± 10.9	36.1 ± 11.2
Sex, females/males	66/12 (85–15)	30/9 (78–22)	14/2 (87–13)
Frequency of extraintestinal symptoms	52 (66)	25 (64)	8 (50)
Frequency of IBS-like symptoms	68 (87)	30 (77)	4 (25)
Frequency of dyspepsia or GER-like symptoms	33 (42)	16 (40)	8 (50)
Multiple food sensitivity	40 (51)	None	3 (20)
Frequency of atopic diseases	27 (35)	7 (18)	4 (25)
DQ2 or DQ8 haplotype	48 (62)	18 (45)	16 (100)

NOTE. Values are n (%).
CD, celiac disease; GER, gastroesophageal reflux; IBS, irritable bowel syndrome; NCGWS, nonceliac gluten/wheat sensitivity.
Bowel habit characteristics in patients with IBS symptoms: NCGWS: diarrhea 44 (65%), constipation 11 (16%), alternating bowel movements 13 (19%). Non-NCGWS control subjects: diarrhea 18 (60%), constipation 3 (10%), alternating bowel movements 9 (30%). CD: diarrhea 3 (75%), alternating bowel movements 1 (25%).

Non-Celiac Wheat Sensitivity"). All authors had access to the study data and reviewed and approved the final manuscript.

Results

Of the 522 patients initially evaluated, 330 patients agreed to follow a wheat-free oligoantigenic diet after clinical and laboratory work-up and careful exclusion of other diagnoses. Of these, 115 patients improved on elimination diet and accepted to undergo a DBPC wheat challenge. In total, 78 of 115 tested positive and did not react to placebo and were consequently included in the study (for details, see Supplementary Figure 1).

Table 1 summarizes the clinical characteristics of the study patients, most of them showing IBS-like symptoms. Similar characteristics were observed in the control group composed of patients with self-reported wheat sensitivity and negative DBPC challenge (non-NCGWS control subjects).

Histologic evaluation of the duodenal mucosa showed that none of the patients with NCGWS or non-NCGWS control subjects had a villus/crypt ratio <3, whereas all CD control subjects showed villous atrophy. $CD3^+$ IELs progressively increased from the non-NCGWS control subjects (14.3 ± 4.2, mean ± standard deviation of IEL number) to patients with NCGWS (19.6 ± 10.7; $P < .03$) and CD control subjects (47.7 ± 23.3; $P < .001$ vs NCWS patients). Figure 1*A* shows the individual numbers of $CD45^+$ immunocytes in the duodenal lamina propria of the 3 study groups: patients with NCGWS had a significantly higher number of lamina propria $CD45^+$ than non-NCGWS control subjects, with values between those of this control group and CD control subjects.

Figure 1*B* shows eosinophil numbers in the duodenal lamina propria of the 3 groups. We found significantly higher eosinophil numbers in patients with NCGWS and in CD control subjects than in the non-NCGWS control subjects. Furthermore, the proportion of cases with eosinophil numbers greater than or equal to the upper normal limit for our laboratory was significantly higher in the NCGWS group than in the non-NCGWS control subjects ($P < .0001$; Fisher test).

In the 33 patients with NCGWS who reported upper digestive tract symptoms (dyspepsia or gastroesophageal reflux–like symptoms), the number of lamina propria eosinophils was significantly higher than in the remaining patients with NCGWS who did not report such symptoms (8.6 ± 2.6 vs 6.8 ± 3.6 per high power field; $P < .01$). Figure 2 shows a representative picture of the duodenal mucosa of 1 of the patients with NCGWS included in the study.

There was a trend toward higher number of duodenal lamina propria $CD3^+$, and $CD8^+$ lymphocytes and mast cells in patients with NCGWS than in non-NCGWS control subjects, and toward lower number of $CD4^+$ lymphocytes, but without a statistically significant difference. Of the 3 study groups, CD control subjects showed the highest number of lamina propria $CD3^+$, $CD4^+$, and $CD8^+$ lymphocytes, and mast cells (data not shown).

Rectal histology of the patients with NCGWS did not demonstrate any features of inflammatory bowel disease (ie, crypt abscesses, granulomas). It was characterized by the presence of lymphoid nodules. Lymphoid follicles were found in 74 of 78 patients with NCGWS and in 26 of 39 non-NCGWS control subjects (chi-square test, 6; $P < .0001$). Furthermore, follicles were significantly larger in patients with NCGWS (median, 350 μm; range, 0–670) than in non-NCGWS control subjects/all control subjects (median, 262 μm; range, 0–430) ($P < .0001$). The patients with NCGWS also showed a higher number of IEL $CD3^+$ lymphocytes and lamina propria $CD45^+$ and eosinophils than control patients (Figure 3). Furthermore, the frequency of cases with eosinophil numbers greater than the upper normal limit for our laboratory was significantly higher in the NCGWS group (73 out of 78) than in non-NCGWS control subjects (17 out of 39; $P < .0001$; Fisher test). Figure 4 shows a representative picture of the rectal mucosa of 1 of the patients with NCGWS included in the study.

No differences were observed between the NCGWS and non-NCGWS control patient groups for lamina propria $CD3^+$, $CD4^+$, $CD8^+$ lymphocytes and mast cells, although there was a trend toward higher values in the NCGWS group than in the non-NCGWS control subjects (data not shown).

In patients with NCGWS, the mean eosinophil infiltration was more than 2.5-fold the upper normal limit in the rectum and almost 2-fold in the duodenum (Figure 5; $P < .0001$). An inverse pattern was seen in the non-NCGWS control subjects, with a tendency toward a higher eosinophil infiltration in the duodenum than in the rectum.

Agreement between the pathologists in the evaluation of the "presence or absence" of the eosinophil infiltration counted in 5 HP fields was good ($K = 0.88$).

Discussion

Although the most common clinical presentation of NCGWS overlaps with IBS, there are no previous studies evaluating colon or rectal histology in patients with NCGWS, and consequently duodenal and rectal mucosa histology in NCGWS have never been compared previously. In this study we showed that mucosal inflammation, both in the duodenum and in the rectal mucosa, is common in patients with NCGWS. Indeed, lamina propria $CD45^+$ cells, representing the "total immunocyte" infiltration, were significantly higher in patients with NCGWS than in the non-NCGWS control subjects at both sites. Furthermore, a higher number of IEL $CD3^+$ lymphocytes was found in the duodenal mucosa of patients with NCGWS than in non-NCGWS control subjects, in accordance with previous studies that reported "epithelial

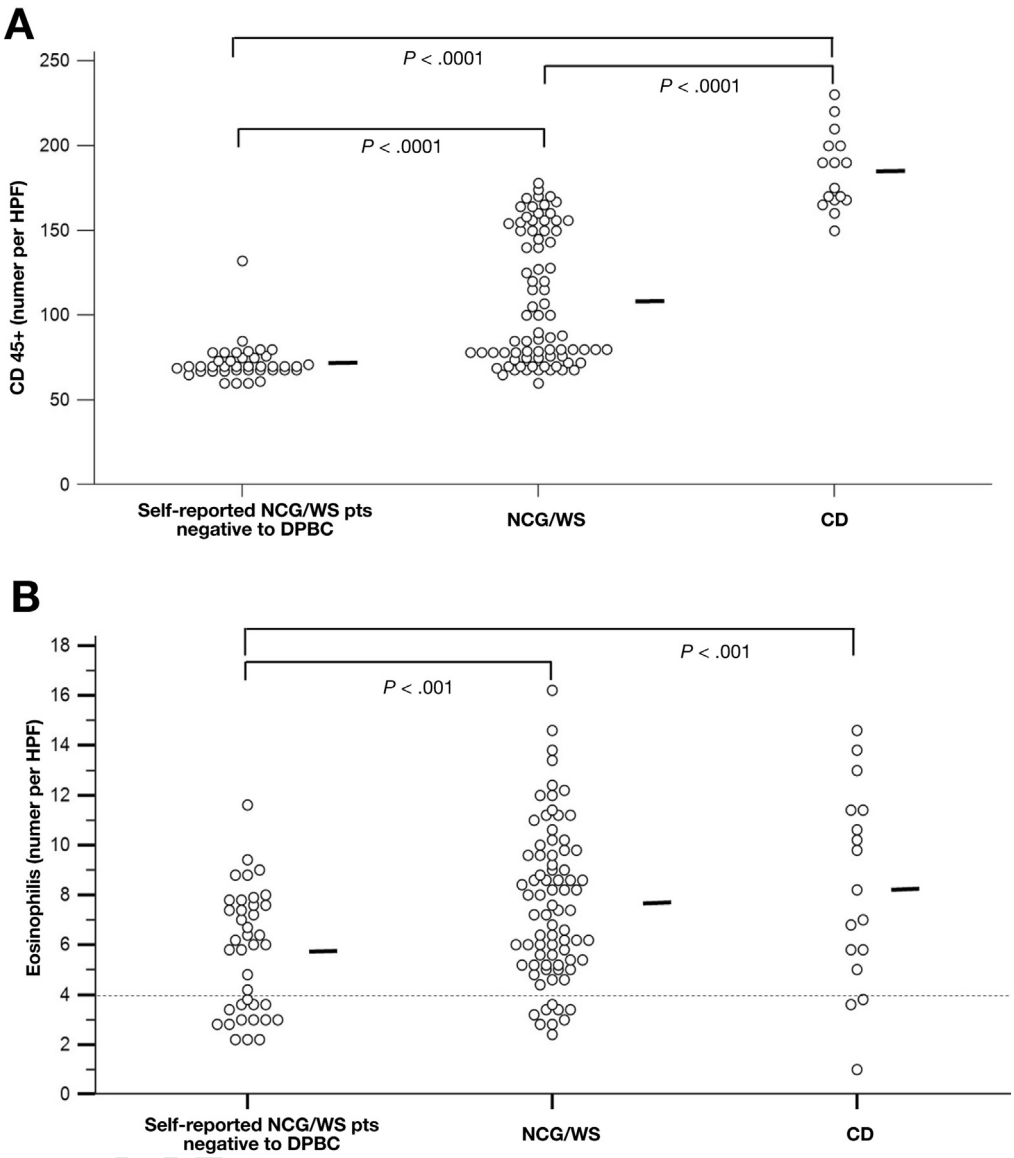

Figure 1. Individual values of CD45 (immunocytes) (*A*) and eosinophil count (*B*) in the lamina propria of the duodenal mucosa in the 3 groups: control subjects negative at the DBPC challenge (non-NCGWS control subjects), NCGWS patients (NCGWS), and celiac disease control subjects (CD). *Horizontal blue bars* indicate the median value. The *dotted line* in *B* indicates the upper normal limit for lamina propria eosinophil count in our laboratory. HPF, high-power field.

lymphocytosis" (Marsh 1 lesion) in the duodenum of a subset of patients with NCGWS.[10] This finding is also in agreement with an endomicroscopy study performed in IBS patients, which revealed $CD3^+$ IEL infiltration immediately after exposure to food antigens.[4]

The most interesting histologic finding in NCGWS was an increase in eosinophils in the lamina propria of the duodenal and rectal mucosa. In both these sites, eosinophil numbers were higher in NCGWS than in the non-NCGWS control subjects. Furthermore, eosinophil numbers in the duodenal mucosa were higher in the patients with NCGWS with dyspepsia than in the patients with NCGWS without upper digestive tract symptoms. Functional dyspepsia is frequently associated with IBS, suggesting that these 2 diseases have a shared pathogenesis[11]; increased eosinophil infiltration has also been observed in the duodenum of a subset of patients with functional dyspepsia[12] and could play a pathogenetic role.[13,14] In these patients, food antigens, including wheat proteins, were hypothesized to initiate a Th2 response driving intestinal eosinophilia.[11] On the basis of our data, it could be suggested that NCGWS (or multiple food sensitivity) is a true diagnosis in a subset of patients with dyspepsia.

Duodenal (and rectal) eosinophils could have a possible pathogenetic role in NCGWS. Previous studies have demonstrated a neurologic dysfunction driven by the production of eotaxin, a chemokine specific for eosinophils, in a murine model.[15] Furthermore, we have shown high levels of eosinophil cationic protein in the stools of patients with IBS with food allergy.[5]

The eosinophil infiltration seemed to be more significant in the rectum than in the duodenum in patients with NCGWS. We recognize that this cannot be considered a specific marker of NCGWS, because eosinophils can be found in the colon and rectal mucosa in several clinical conditions, such as inflammatory bowel diseases and CD, among others. However, these clinical conditions have clinical, endoscopic, serologic, and histologic aspects markedly different from NCGWS, and we would

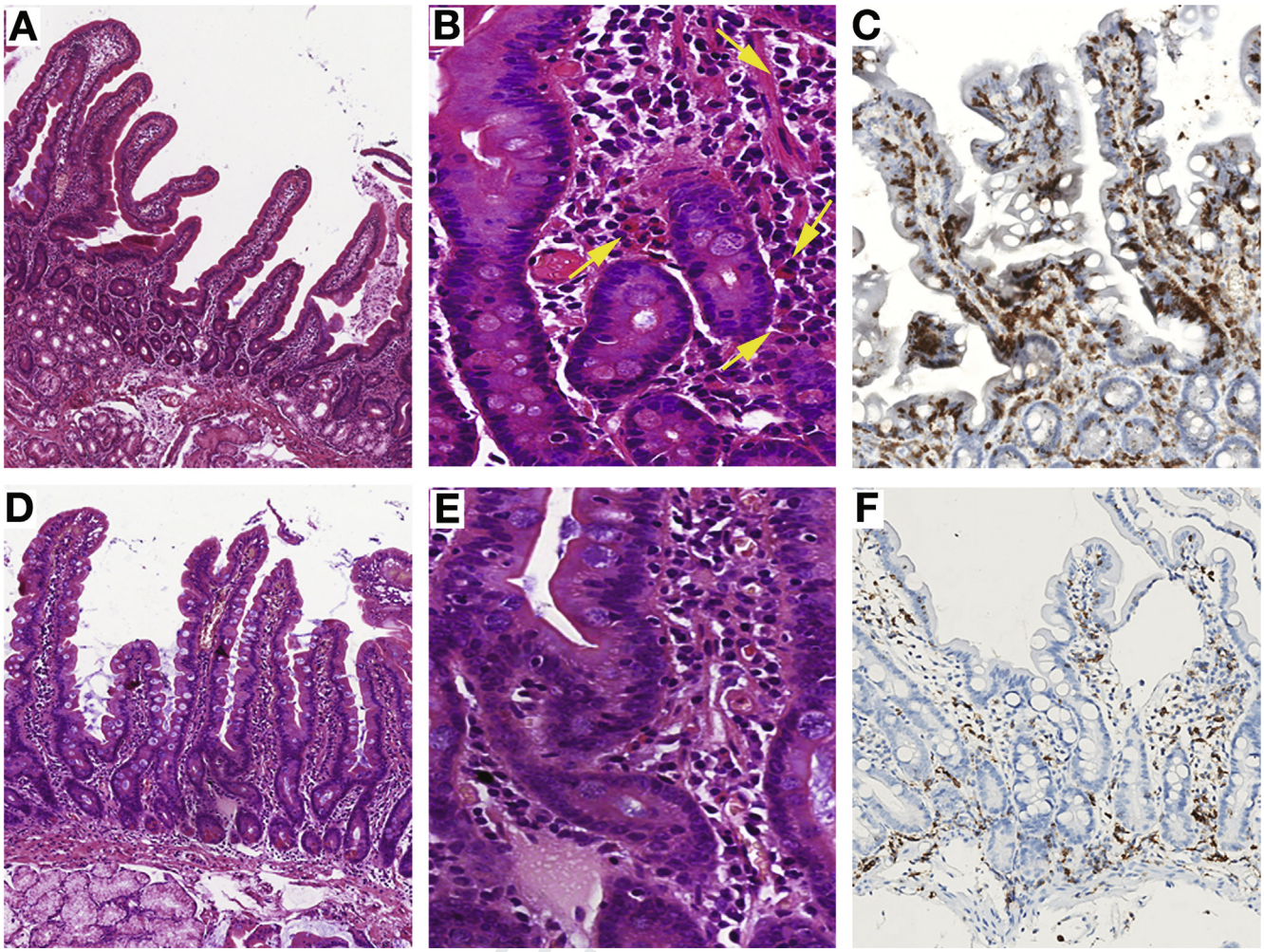

Figure 2. Duodenal mucosa of a NCGWS patient (*A–C*) compared with a non-NCGWS patient (control subject) (*D–F*). Villi had a substantially normal structure (*A*, *D*). The eosinophil (some are indicated with a *yellow arrow*) count in the lamina propria was slightly higher in the NCGWS patients (*B*) than in control subjects (*E*), as was the overall immunocyte count, assessed with CD45 immunohistochemical staining (*C*, *F*). (Original magnification ×40 [*A*, *C*], ×200 [*B*, *E*], ×100 [*C*, *F*].)

suggest that in clinical practice, subjects showing an IBS clinical presentation and mucosa eosinophil infiltration should be recommended to commence an elimination diet with a subsequent wheat challenge.

Other histologic findings in the rectal mucosa of the patients with NCGWS were noteworthy. About 95% of patients, in fact, showed lymphoid follicles, which were significantly larger than in the control subjects ($P < .0001$). Again, lymphoid follicles can be considered a "normal finding" in rectal mucosa, but in our experience, the presence of large follicles is associated to non-IgE-mediated food allergy,[16] a condition that we consider to be one of the pathogenetic factors of NCGWS.[17] On the whole, it can be hypothesized that not only eosinophils could play a pathogenetic role in NCGWS, and that a complex immunologic response involving both innate and acquired immunity may be responsible for this disease.[9,18]

Our study has some limitations. Our findings cannot be attributed to all "people who avoid gluten."[19] We studied patients referred to tertiary centers with experience in treating NCGWS, and this factor led to a selection bias. Our results must not be extended to all self-treated or diagnosed patients with NCGWS. NCGWS cannot be considered a homogeneous condition, but rather an "umbrella" term that includes various conditions with different types of pathogenesis.[17] Some relevant studies have underlined a prevalent pathogenetic role for the fermentable oligo-, di-, and mono-saccharides and polyols, instead of gluten, in self-reported NCGWS subjects.[20,21] In our opinion, those studies involved a "different" self-reported NCGWS patient population, with less prominent immunologic characteristics than the ones evaluated here and in previous studies.[10,22] Thus, the histology findings that we found in our patients likely characterize patients with NCGWS who have a high level of immunologic activation and, perhaps, a non-IgE-mediated form of wheat allergy. A possible selection bias of our study population is also suggested by the high rate of positive DBPC challenges, which contrasts with previous lower percentages.[23]

Evaluation of the eosinophil infiltration was performed by means of the simple hematoxylin-eosin

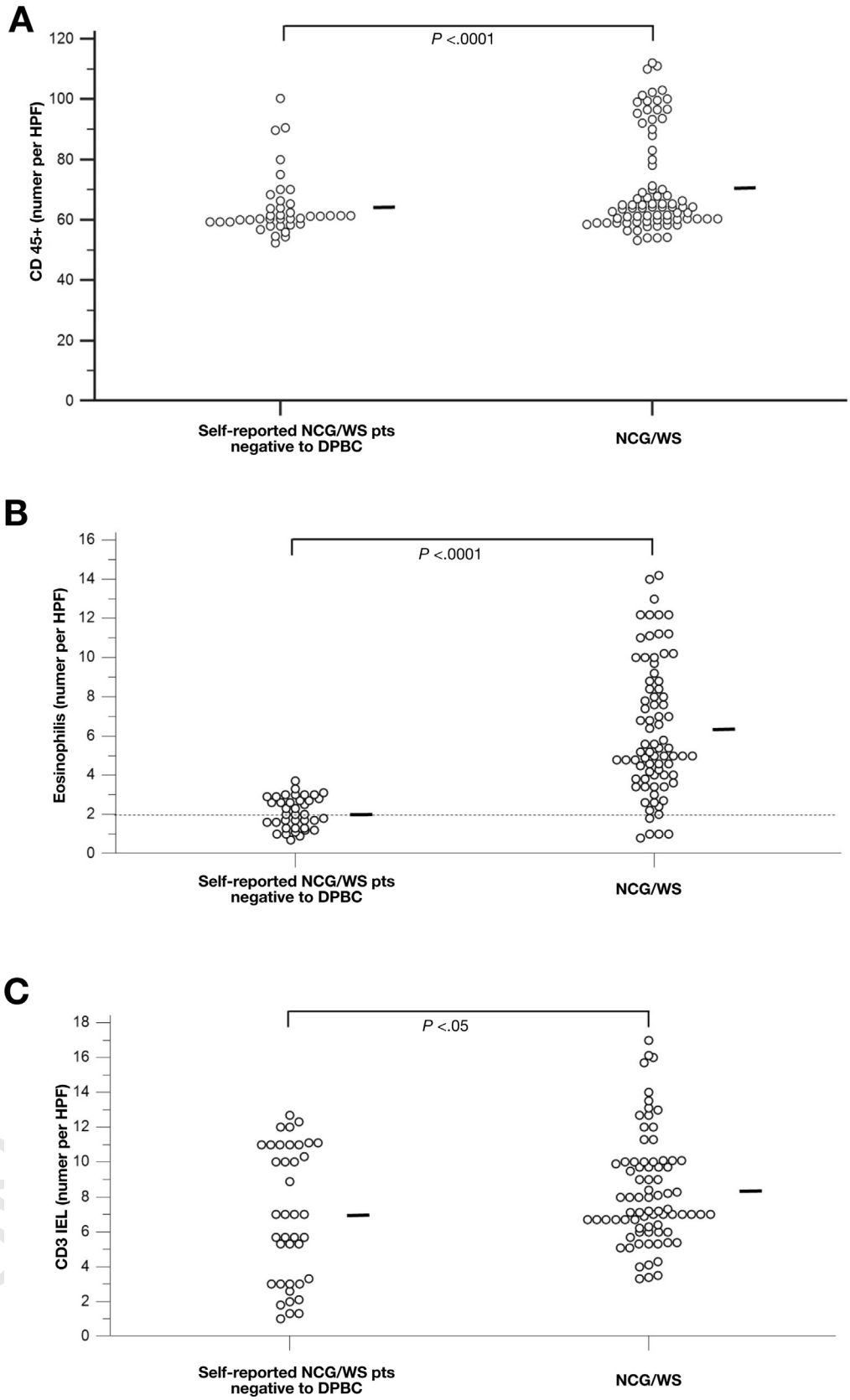

Figure 3. Individual values of lamina propria $CD45^+$ lymphocytes (*A*) and eosinophils (*B*) and intraepithelial $CD3^+$ cells (*C*) in the rectal mucosa of patients with NCGWS (Group 1) and in non-NCGWS control subjects (Group 2). *Horizontal blue bars* indicate the median value. The *dotted line* in *B* indicates the upper normal limit for lamina propria eosinophil count in our laboratory. HPF, high-power field.

method, whereas immunohistochemistry is reported to be more accurate. We did not perform "functional studies" but a simple cell count; other immune cells probably play a relevant role in the intestinal inflammation in NCGWS. We excluded patients on steroids or nonsteroidal anti-inflammatory drugs but included patients on protonic pump inhibitors. We did not include "asymptomatic" control subjects in the

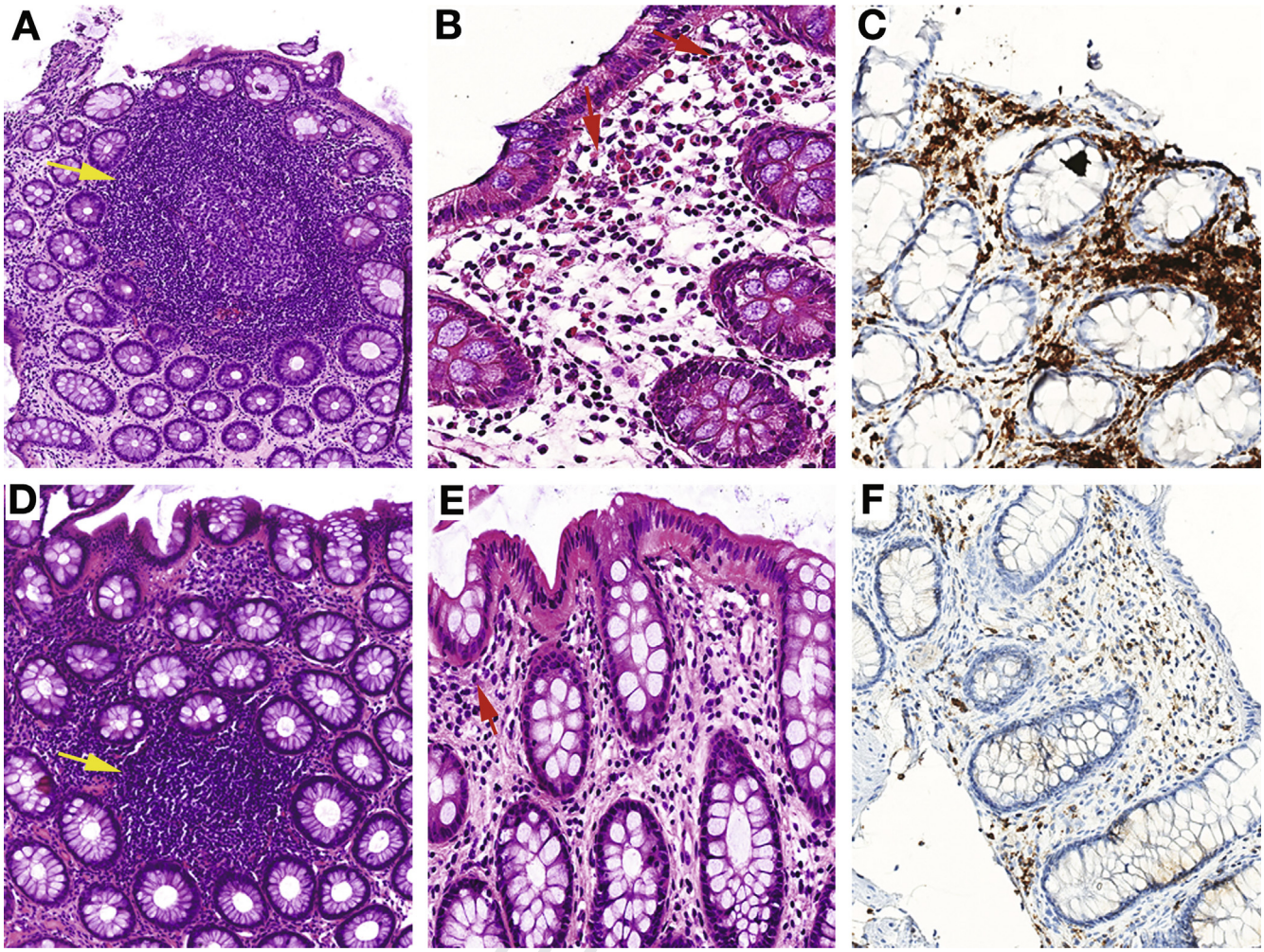

Figure 4. Rectal mucosa of a NCGWS patient (*A–C*) compared with a non-NCGWS control patient (*D–F*). Rectal mucosa in the patients with NCGWS frequently showed lymphoid follicles (*yellow arrow*), often with an activated germinal center (*A*); in control subjects lymphoid follicles were less frequently observed, and with a smaller mean diameter (*D*) than those of patients with NCGWS. Eosinophil density (some are indicated with a *red arrow*) in the lamina propria was significantly higher in patients with NCGWS (*B, E*), as was the overall immunocyte count (*brown-stained cells*), assessed with CD45 immunohistochemical staining (*C, F*). (Original magnification ×100 [*A, C*], ×200 [*B, E*], ×200 [*C, F*].)

study, because it is difficult to select them in such cases.

The main strength of the study is that this is the first prospective study to search for immunohistochemistry modifications in both the duodenal and rectal mucosa of patients with NCGWS. For the first time, we identified histologic markers useful to recommend a wheat-free diet and subsequent challenge in self-reported NCGWS. Furthermore, we showed the rectum is an important site of mucosal inflammation in NCGWS. Our data are in agreement with the clinical aspects of NCGWS, which is very often characterized by alterations in bowel motility and an overlap with IBS. This clinical observation is in line with a higher eosinophil infiltration in the rectal mucosa than in the duodenum. Interestingly, an inverse finding was observed in the control subjects and thus the rectal eosinophil infiltration seems specific to the "subgroup of patients with IBS-like symptoms" secondary to NCGWS. Furthermore, our results show that the patients with NCGWS suffering from upper gastrointestinal symptoms also had a higher eosinophil number in the duodenal mucosa than those without dyspepsia. These results could suggest a real role for eosinophils in the pathogenesis of NCGWS. Furthermore, their "homing" in

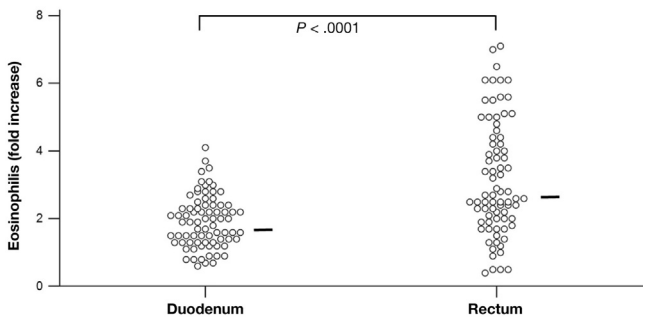

Figure 5. Comparison of the lamina propria eosinophil infiltration in the duodenum and rectum of the patients with NCGWS. The individual values were calculated as fold increases over the upper normal limit. *Horizontal blue bars* indicate the mean value.

the colon, instead of in the duodenum or other gastrointestinal tracts, could probably determine the specific symptoms reported by patients, which involve either the upper or lower gastrointestinal tract. From a clinical standpoint, these data suggest a role for rectal biopsies while considering an elimination diet in subjects with suspected NCGWS.

In conclusion, NCGWS could be considered an inflammatory condition of the entire intestinal tract and the eosinophil infiltration may represent a key candidate player in the pathogenesis of NCGWS.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at https://doi.org/10.1016/j.cgh.2018.08.043.

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Acknowledgments
The authors thank English native speaker Carole Greenall for revising the text. Q3

Conflicts of interest
The authors disclose no conflicts. Q4

Funding
The study was supported by the Italian Foundation for Celiac Disease Grant for Project 013/2014. The sponsor had no role in the study design, collection, analysis, and interpretation of the data. Q1 Q5

Supplementary Appendix 1

Exclusion of Celiac Disease Diagnosis

Before entering the study, patients were instructed to eat foods containing wheat, consuming at least 5 slices of wheat bread per day (about 8 g of gluten) for 4 weeks. At the end of this period, all patients underwent assays for serum antitransglutaminase IgA, antideamidated gliadin peptides IgG, and antigliadin IgA and IgG, performed using commercial kits (Eu-antitransglutaminase IgA, and antigliadin IgA and IgG, Eurospital Pharma, Trieste, Italy; Quanta-Lite Gliadin IgG II, Inova Diagnostics, San Diego, CA). Patients were also typed for HLA-DQ phenotypes by polymerase chain reaction, using sequence-specific primers with a rapid detection method (DQ-CD Typing Plus kit, BioDiaGene, Palermo, Italy). Patients positive for the DQ2 and/or the DQ8 haplotypes also underwent duodenal mucosa biopsy, regardless of the results of the CD-specific antibody assay.

CD diagnosis was excluded when DQ2 and/or DQ8 haplotypes were absent, or when antitransglutaminase IgA and anti-DPG IgG were negative and duodenal histology showed a normal villus/crypt ratio ($\geq$3). Furthermore, CD diagnosis was not excluded if patients were positive at antiendomysium assay of the culture medium of the duodenal biopsies, even if the villus/crypt ratio in the duodenal mucosa was normal. Consequently, these patients were not included in the NCWS group.

Exclusion of Inflammatory Bowel Disease Diagnosis

IBD diagnosis was excluded when serum C-reactive protein, erythrocyte sedimentation rate, and white blood cell count were normal in repeated examinations, performed when the patients were symptomatic. Furthermore, all patients underwent abdominal ultrasound evaluation of the intestinal loop and those with ultrasound signs of suspected IBD were excluded. Patients with a clinical history of suspected IBD (ie, presence of rectal bleeding or hematochezia) also underwent a complete ileocolonoscopy. IBD diagnosis was excluded in these when both endoscopy and histology were negative.

Elimination Diet and Double-Blind Placebo-Controlled Challenge

On entering the study, all patients commenced a standard elimination diet, which excluded wheat, cow's milk, eggs, tomato, and chocolate. Patients self-reporting food hypersensitivity were also asked to avoid ingestion and/or contact with the foods causing symptoms. Food diaries were kept during the elimination diet period to assess dietary intake and adherence to the diet. After 4 weeks of elimination diet, DBPC challenges were performed, with the reintroduction of a single food at a time. Patients were randomized to receive either the "active food" or the placebo, according to a computer-generated order determined by an observer not involved in the study.

In the case of wheat, the DBPC challenge was performed with sachets of flour coded A or B containing wheat flour or rice flour, respectively. Sachets A or B were given for 2 consecutive weeks, and then after 1 week of washout patients received the other sachets for another 2 weeks (crossover design). Wheat challenge was performed by administering a daily dose of 80 g of flour, which was dissolved and cooked by the patients themselves. Wheat sachets contained 6.5 g of gluten.

DBPC for cow's milk was performed by administering capsules coded A or B, containing milk proteins (casein from bovine milk, lactalbumin, lactoglobulin, daily dose 6 g, equal to about 200 mL of cow's milk) or xylose, respectively. A total of 6 capsules per day were given 3 times daily, away from meals.

The codes of the sachets and capsules were broken only at the end of the study and the investigators did not know their contents during the study period. Challenges for other foods in patients with suspected multiple food hypersensitivity were performed in an open fashion.

During all phases of the study, including the challenge period, the severity of symptoms was recorded: patients completed a 100-mm visual analog scale (with 0 representing no symptoms), which assessed overall symptoms and the specific symptoms they each reported.

The challenges were stopped when clinical reactions occurred for at least 2 consecutive days (increase in visual analog scale score $>$30: both for IBS-like symptoms [onset of abdominal discomfort or pain, associated with a change in stool frequency and/or stool appearance] and for extraintestinal symptoms). Challenges were considered positive if the same symptoms that had been initially present reappeared after their disappearance on elimination diet and if the visual analog scale score was $>$30 when compared with any eventual increase determined during the placebo administration.

Supplementary Appendix 2

Histology and Immunohistochemistry

Histopathologic analysis was performed on formalin-fixed, paraffin-embedded duodenal and rectal biopsy specimens, at the Anatomic Pathology Section of the Department of Sciences for the Promotion of Health and Mother and Child Care, University of Palermo, Italy.

For duodenal specimens, 4-μm-thick sections were routinely stained with hematoxylin-eosin to assess architecture, villus/crypt ratio, crypt hyperplasia, edema, degree of inflammatory infiltration of the lamina propria, and eosinophil density.

For rectal specimens, 4-μm-thick sections were routinely stained with hematoxylin-eosin to assess

architecture, edema, degree of inflammatory infiltration of the lamina propria, presence and number of lymphoid nodular aggregates, and eosinophil density.

Eosinophil density was evaluated by counting the total number of cells per 5 high-power fields, in accordance with Walker and Talley.[1] Eosinophils in the lamina propria were counted on the hematoxylin-eosin slides and expressed as eosinophils per high-power field (×40). In the duodenum, lamina propria eosinophils per 10 high-power fields were counted and the value recorded was the mean of the count in these 10 fields.

The composition of the inflammatory infiltrate in the lamina propria of both the duodenum and rectum was assessed and immunohistochemical staining was used to count and classify the inflammatory cells. The primary antibodies used were: $CD3^+$ T lymphocytes, $CD4^+$ T helper lymphocytes, $CD8^+$ cytotoxic lymphocytes, tryptase for mast cells, and $CD45^+$ cells.

Immunohistochemical staining was performed with the BenchMark XT automated slide staining system (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's instructions, using the following primary antibodies: CD3 (rabbit monoclonal, clone: 2GV6), CD4 (rabbit monoclonal, clone: SP35), CD8 (rabbit monoclonal, clone: SP57), CD45-LCA (mouse monoclonal, clone: RP2/18), and tryptase (mouse monoclonal, clone: G3). Negative control subjects without primary antibodies were included in each immunohistochemical run. The slides were analyzed under a Leica-DM2000 optical microscope (Leica Microsystems, Exton, PA) using Leica ×4 SL, ×10 SL, HI PLAN ×20/0.40, HI PLAN ×40/0.65, HI PLAN ×63/0.75, and PL FLUOTAR ×100/1.30 objectives. Microphotographs were obtained using a Leica MC120 HD camera (Leica Microsystems).

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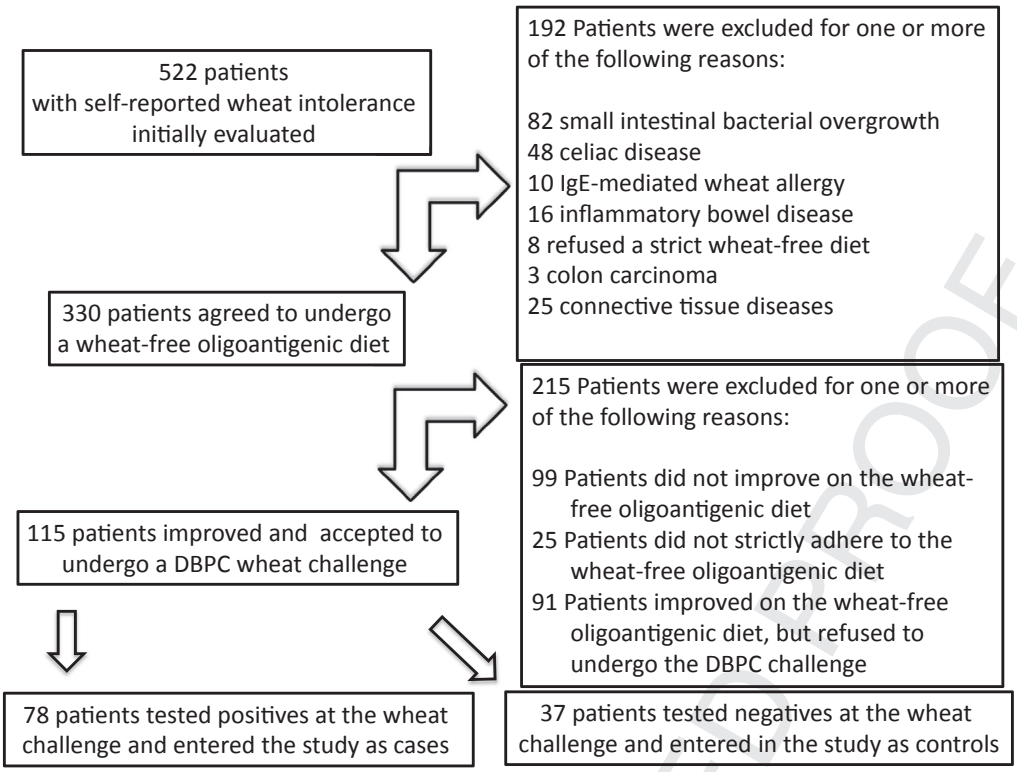

Supplementary Figure 1. ■■■